5-2008

An Analysis of COX-2 Derived Prostaglandin E2 (PGE2) in the Alpha-1 and Alpha-2 Adrenoreceptor-Mediated Responses of Thermally Classified Neurons in the Anterior Hypothalamus

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An Analysis of COX-2 Derived Prostaglandin E_2 (PGE_2) in the Alpha-1 and Alpha-2 Adrenoreceptor-Mediated Responses of Thermally Classified Neurons in the Anterior Hypothalamus

A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelors of Science in Neuroscience from
The College of William and Mary

by

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Williamsburg, VA
April 22, 2008

Abstract

In vivo evidence demonstrates that the initial febrigenic signal from the periphery is communicated to the anterior hypothalamus (AH), the thermoregulatory center of the brain, via ascending projections of the hepatic vagus nerve. The subsequent release of Norepinephrine (NE) in the AH is a key intermediary of the febrile response, resulting in two distinct phases mediated by α1 and α 2 adrenoreceptors (AR). Activation of the α1 AR produces a PGE_2- independent rise in body temperature, whereas α2 activation yields a biphasic response; hypothermia followed by a PGE_2-dependent temperature increase (Feleder et al., 2007). What remains unknown is how these ARs modify the firing rate of thermoregulatory neurons within the AH to drive these phases and which cyclooxygenase (COX) isozyme is responsible for PGE_2 production. The action of NE in the AH was tested in the present study with the selective COX-2 inhibitor Meloxicam (0.1-10 μM) by recording single-unit activity of AH neurons in a tissue slice preparation from the adult male rat, in response to temperature and the selective α1 AR agonist Cirazoline (1 μM) or the selective α2 AR agonist Clonidine (1 μM). All neurons were classified as either warm-sensitive or temperature insensitive. Warm-sensitive neurons responded to Cirazoline with a decrease in firing rate, while temperature insensitive neurons showed a firing rate increase. These findings strengthen the role of the α1 AR in quickly driving set-point temperature into a hyperthermic range to initiate fever in a PGE_2- independent fashion. In contrast, warm-sensitive neurons responded to Clonidine with an increase in firing rate, while temperature insensitive neurons showed a firing rate decrease. This indicates the α2 AR may initially be driving an opposing hypothermia, but COX-2 inhibition eliminated late phase responses after α2 AR activation, suggesting that PGE_2 from this pathway is responsible for sustaining the fever initiated by α1 AR.

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Introduction

Fever and the Hypothalamus

A fever is the temporary elevation of set-point body temperature usually one to two degrees above normal, which is about 37˚ Celsius in humans. The fever is a hallmark indicator of the immune system fighting an infection and presumably increases the host's chances of survival by fostering a hostile environment for the invading pathogen. As well, the slight increase in temperature enhances certain immune system, endocrine, and metabolic functions to hasten defense processes (Roberts, 1991). The maintenance of temperature during a fever must be very precise; prolonged, unmanaged adjustments can be fatal, as so many cellular processes depend on a narrow window of temperature in which to properly function. Thermoregulation is thus of utmost significance and investigation may improve clinical understanding of mechanisms behind certain illnesses, hot-flashes, and immune system function.

Achieving this rise in temperature involves a complicated series of coordinated autonomic, endocrine, and behavioral events, all of which are ultimately controlled by the hypothalamus, the body's central thermostat (Boulant, 1992). The hypothalamus is centrally located in a region known as the diencephalon, at the base of the forebrain just above the brainstem. It is bound by the optic chiasm rostrally and midbrain tegmentum caudally, forming the floor and ventral wall of the third ventricle. This unique orientation allows the hypothalamus to function as an autonomic integrator; comparing sensory inputs from visceral, somatic, chemoral and humoral pathways with those from higher areas such as the cerebral cortex, amygdala, and hippocampus (Purves et al., 2004). Noting discrepancies between these varying inputs and serving as a reference, the

hypothalamus has control over a variety of effector systems to establish homeostasis and elicit necessary behavioral responses.

The anterior hypothalamus (AH) is the region of chief importance in thermoregulation, as it receives input from skin and spinal thermoreceptors to monitor temperature throughout the body (Boulant, 2000). Within this area are distinct populations of neurons that can be defined by their inherent ability to respond to changes in temperature. This classification is typically done by recording action potential firing rate in response to induced changes in temperature. A thermosensitivity coefficient (*m,* impulses$\cdot s^{-1}\cdot{}^{\circ}C^{-1}$) can be determined from the regression line slope of a plot comparing firing rate and temperature. A wide body of *in vivo* research has correlated changes in temperature with neuronal activation and initiation of thermal responses; these criteria can be used to determine a neuron's thermal properties (Boulant and Hardy, 1974).

The majority of AH neurons are temperature insensitive (I; $m < 0.80$); that is their firing rate remains relatively constant despite changes in temperature. Constituting about 70% of the neurons in the AH, the consistent input of insensitive neurons is believed to function as reference point by which to establish a temperature set-point (Boulant, 2006). Approximately 20% are warm-sensitive neurons (W; $m \geq 0.80$) who robustly increase their firing rate in response to warming. This distinction between warm-sensitivity and insensitivity is determined by differences in ionic conductance during depolarizing pre-potentials, rather than temperatures effects on resting membrane potential (Zhao and Boulant, 2005). After an action potential, there is a fast hyperpolarization followed by a delayed depolarization which will lead to threshold and generation of a subsequent action potential. Insensitive neurons show little change in the depolarizing prepotential, thus the

interspike period remains consistent, generating strong pacemaker activity (Griffin et al., 1996). On the other hand, warm-sensitive neurons show an increase in the depolarizing prepotential in response to temperature increases, which is likely due to inactivation of an outward hyperpolarizing potassium current, referred to as I_A (Griffin et al., 1996). As a result, warm-sensitive neurons can reach threshold much more rapidly, shortening the interspike period, leading to an increased firing rate.

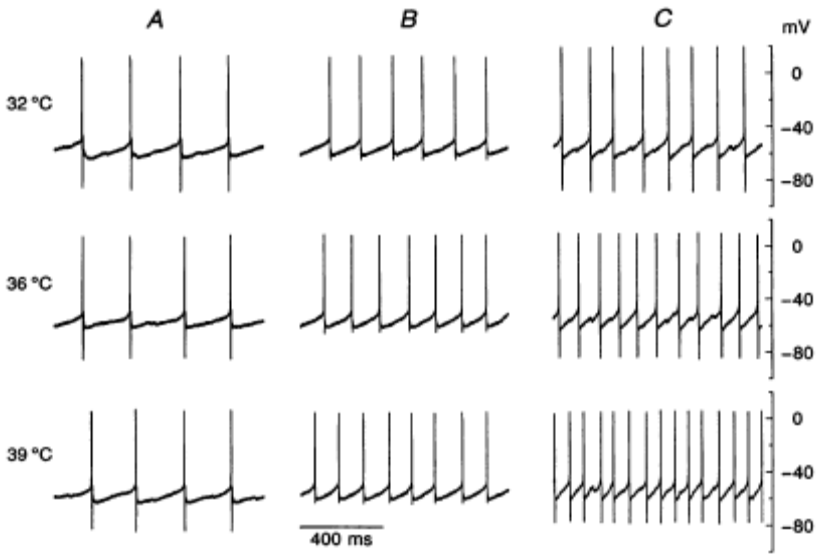

Figure 1, from Griffin et al. (1996). Intracellular recordings illustrating differences between interspike interval for insensitive (A; *m* = 0), slightly warm-sensitive (B, *m* = 0.5), and warm-sensitive (C, *m* = 1.1) neurons during induced increases in temperature.

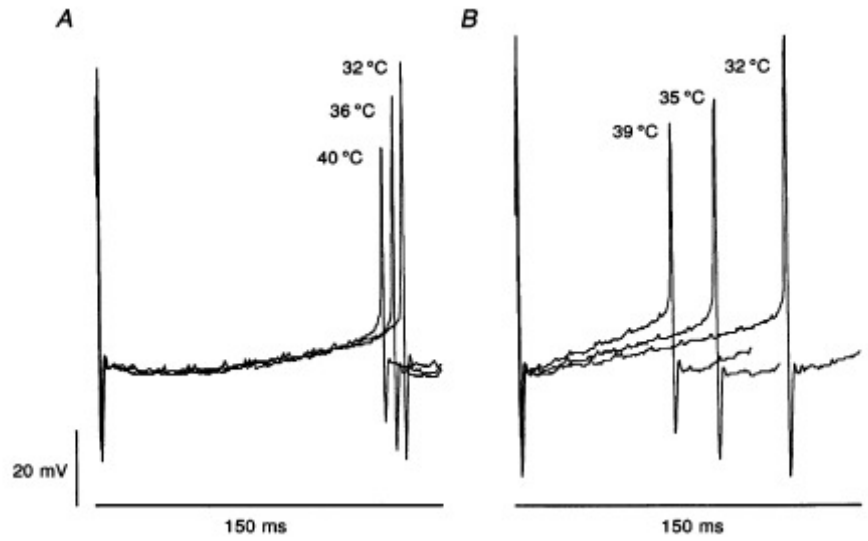

Figure 2, from Griffin et al. (1996). Intracellular recordings of insensitive (A) and warm-sensitive (B) neurons in response to increases in temperature. Note in B the shortening of the depolarizing prepotential as temperature increased.

In addition to different electrical properties, insensitive and warm-sensitive neurons can be characterized by unique morphology in the AH. Warm-sensitive neurons orient their dendrites medially to the third ventricle and laterally to the medial forebrain bundle (Griffin et al., 2001). This positioning is believed to aid the integration of the thermal input from two higher and lower afferent pathways (Boulant, 2006). In contrast, insensitive neurons are situated parallel to the midline third ventricle, away from lateral inputs that synapse on warm-sensitive neurons. The lack of thermal input is consistent with the function of insensitive neurons; pacemakers to provide a constant reference signal.

Hammel's Model of Thermoregulation

The model proposed by Hammel (1965), which has endured as a simple, yet valuable explanation of hypothalamic thermoregulatory interactions, must be examined in

order to understand how these neurons can work to raise body temperature during a fever. It is centered around the action of warm-sensitive and insensitive neurons, as well, it details heat-loss effector neurons and heat-production effector neurons. The insensitive and warm-sensitive neurons send antagonistic, synaptic inputs to these effector neurons which control thermoregulatory responses. Warm-sensitive neurons can excite heat-loss effector neurons and inhibit heat-production effector neurons; insensitive neurons inhibit and excite these effector neurons, respectively. A temperature set-point is established when the firing rate of insensitive neurons matches that of warm-sensitive neurons. Sensing an increase in temperature from afferent inputs, warm-sensitive neurons increase their firing rate relative to the insensitive neurons. This deviation from set-point would stimulate heat-loss effector neurons, which could initiate responses such as panting, sweating, and vasodilatation. During cooling, the firing rate of warm-sensitive neurons would decrease while firing rate of insensitive neurons would remain relatively unchanged. With this comparative shift of firing rates, the insensitive neurons would inhibit heat-loss effector neurons and excite heat-production effector neurons, which may promote processes of shivering, vasoconstriction, or thermogenesis via uncoupling of oxidative phosphorylation pathways. It is important to note that each thermoregulatory response has its own set-point, thus different responses will be initiated at different temperatures.

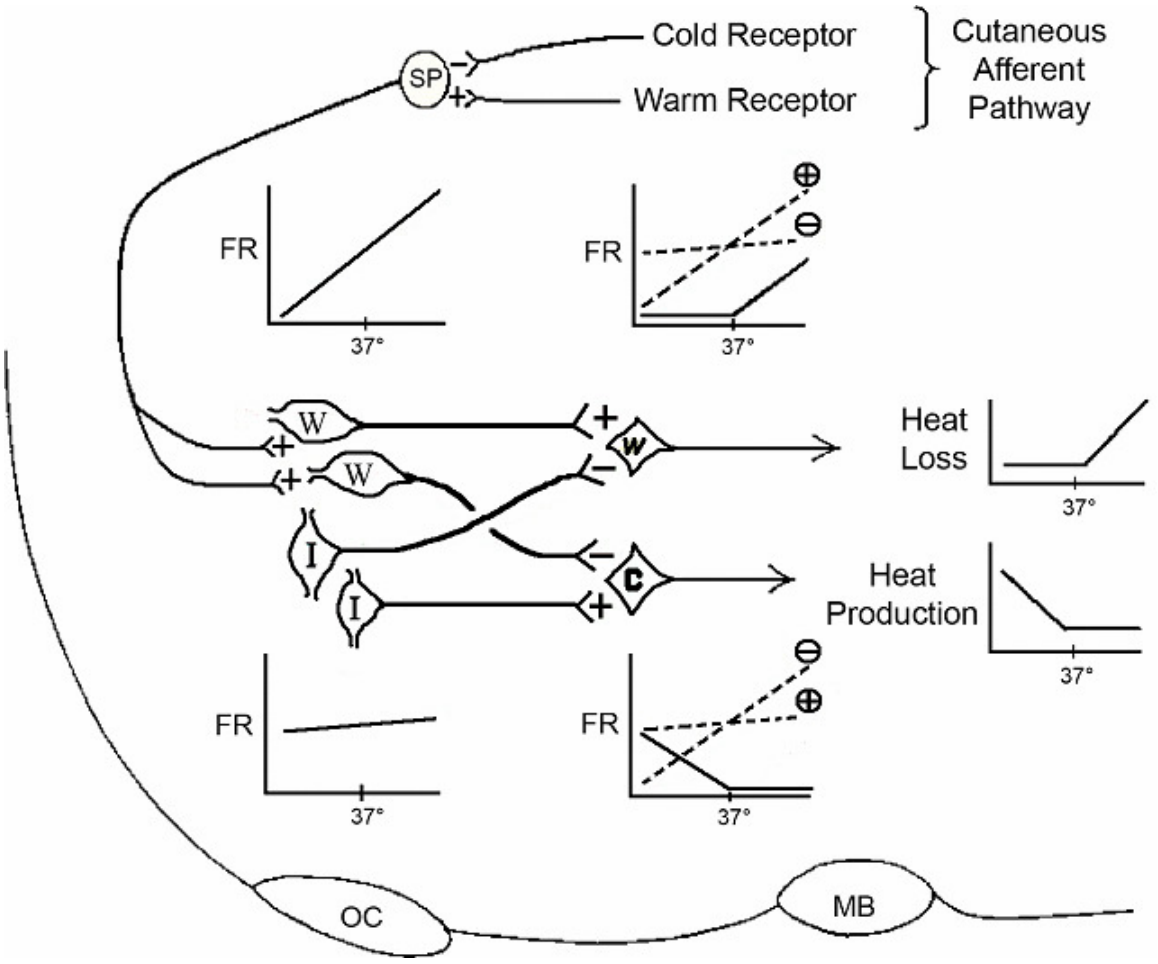

Figure 3, adapted from Boulant (2006). Hammel's model of thermoregulation. The two neurons labeled W are warm-sensitive, the other two labeled I are insensitive. They send antagonistic synapses with warm and cold effector neurons, indicated by diamond shaped W or C neurons, respectively. A + or – indicates an excitatory or inhibitory effect. See above text for details. SP: dorsal horn spinal neuron; OC: optic chiasm; MB: mammilary body, FR: firing rate.

The presence of pyrogens or endotoxins can also affect the activity of AH thermoregulatory neurons, as is the case during a fever. The mediators of these toxins will inhibit the activity of warm-sensitive neurons; this relative decrease compared to the insensitive neurons would inhibit heat-loss responses while enhancing heat-production responses, and the set-point temperature would be elevated (Boulant, 2000). With pyrogens present, all responses will have elevated set-point temperatures, presumably, once levels decrease, the firing rate of warm-sensitive neurons will return to their previous, higher level. This would enhance heat-loss responses and bring body temperature back down to normal.

Initiation of the Fever Response

The immune system responding to an invading pathogen, through a reaction to an endotoxin such as lipopolysaccharide (LPS), is responsible for communicating with the hypothalamus to adjust set-point temperature as described above. The traditional view has been that pathogens stimulate phagocytes to synthesize and release pyrogenic cytokines into the blood stream. Cytokines, as an endocrine signal, are then transported in the circulation and move through the blood-brain barrier (Banks et al, 1995), where their arrival in the AH will induce the expression of Cyclooxygenase-2 (COX-2) and subsequent synthesis of Prostaglandin E2 (PGE_2), a well-established a fever mediator (Blatteis and Sehic, 1997).

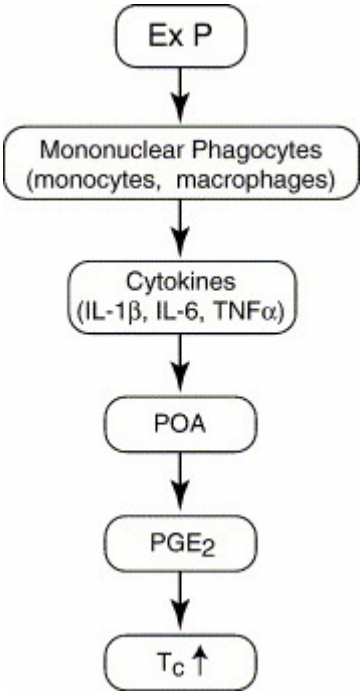

Figure 4, from Blatteis (2005). A schematic highlighting the events of the "classic" fever response. ExP: external pyrogen; IL: interleukin; TNF: tumor necrosis factor; POA: preoptic-anterior hypothalamus; $T_c\uparrow$: core temperature rise.

However, this traditional mechanism of the cytokine-induced febrile response has been re-evaluated because of time course discrepancies in the sequence of events. What has been overlooked is that the production of these signals, which affect transcription rates and gene expression, is a slow process, happening on the order of hours. The appearance of circulating cytokines lags the onset of the febrile response when LPS is intravenously injected, as well; the expression of COX-2 only appears well after the onset of fever (Blatteis, 2005). Additionally, cytokine levels remain very high once the fever has abated and the transport of cytokines across the blood-brain barrier remains very controversial. Evidence now clearly establishes that the initial febrigenic message from the immune system is conveyed to the hypothalamus via a much faster means; a neural

route. The hepatic division of the parasympathetic vagus nerve initially was implicated in this regard, as its surgical removal or destruction prevented the prompt generation of a fever in response to LPS (Simons et al, 1998; MohanKumar et al, 2000). Further research has supported these findings, with the liver and vagus emerging as central intermediaries in the recently elucidated endotoxic febrile response.

Upon arrival in the liver, injected LPS is taken up by Kupffer cells (Kc) and a complement cascade is activated. Kc are unique because they express constitutive levels of both COX-1 and COX-2 enzymes and have PGE_2 available for quick, almost instantaneous release (Blatteis, 2000). The complement promotes Kc to release PGE_2, which stimulates hepatic vagal afferents possessing Prostaglandin E receptors (EP) that project to the AH (Li et al., 2006; Wieczorek and Dunn, 2006). Noradrenergic afferents of the vagus travel from the brainstem through the ventral noradrenergic bundle and synapse in the AH, where there are numerous noradrenergic terminals (Kumar et al, 2007). It is the release of Norepinephrine (NE) via these autonomic routes which mediates the febrile response to peripheral LPS in the AH (Feleder et al., 2007). NE acts at adrenoreceptors (AR) in the AH to initiate two distinct thermal responses; a rapid, initial hyperthermia independent of PGE_2 and a later one that is dependent on COX-2 induction and PGE_2 synthesis (Feleder et al, 2004). The ARs and prostaglandins will be analyzed separately in depth to better understand their intricate relationship in the hypothalamus during fever.

Norepinephrine as a Fever Mediator: the Role of Adrenoreceptors

Adrenoreceptors are G-protein coupled receptors (GPCR) widely distributed in the CNS; they are the targets of NE and epinephrine and are typically involved in autonomic responses. There are two families of AR, α and β, which can further be broken down into various subtypes. Binding of NE at α1 ARs stimulates Phospholipase C (PLC) activity, which catalyzes the hydrolysis of phosphatidylinositol (PIP_2), resulting in formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is an important regulator of intracellular calcium levels by binding to selective channels of the endoplasmic reticulum. DAG remains in the inner membrane leaflet and can go on to stimulate a variety of other pathways. Typically in the CNS, activation of α1 ARs will result in depolarization and an increase in firing rate, as well as increasing sodium-potassium pump activity to restore concentration gradients (Mallick et al, 2000). In contrast to α1 ARs, α2 ARs are GPCR which act to reduce PLC activity. As well as indirectly lowering Ca^{2+} levels, the α2 AR can also inhibit adenylate cyclase, subsequently reducing cyclic AMP (cAMP) production.

These two AR subtypes have different second messenger pathways, which may indirectly explain differences in the thermal responses mediated by each receptor. In fact many neurons possess mixtures of both α1 and α2 ARs in their synaptic membranes, thus adding another level of complexity and more elaborate control of neurotransmission (Siegel et al., 1999). The application of NE in the AH will increase the frequency of spontaneous, miniature inhibitory postsynaptic currents (mIPSCs) via the α1 AR and likewise decrease the frequency of IPSCs via the alpha-2 AR (Kolaj and Renaud, 2007). This observation indicates that these two ARs can modify rapid, GABA-mediated

inhibitory transmission and thus have a central role in regulating excitability within this noradrenergic network of thermoregulatory neurons.

Application of Cirazoline *in vivo*, a NE agonist that acts selectively at the α1 AR, produced a quick rise in core body temperature independent of PGE_2 that attenuated within hours. It appears activation of the α1 AR is important in the genesis of fever; it has been demonstrated that Cirazoline directly augments the firing rates of thermosensitive neurons within the AH *in vitro*, a decrease and increase in warm-sensitive and insensitive neurons respectively (Imbery et al., 2008). These contrasting responses would activate heat-production effector neurons according to the Hammel's thermoregulatory model.

The application of the selective α2 AR agonist Clonidine *in vivo* produced a biphasic response that directly correlated with levels of PGE_2; an initial temperature fall followed by a later temperature rise corresponding to increased PGE_2 levels (Feleder et al, 2004). PGE_2 has been shown to increase and decrease the firing rates of insensitive neurons and warm-sensitive neurons respectively, which would correspond to heat production according to Hammel's model (Ranels and Griffin, 2003). It had initially been accepted that the α2 AR mediates only a hypothermic response (Quan et al, 1992), but the latter temperature rise observed may be a result of a longer experimental recording duration. Treatment with a selective COX-1 inhibitor had minimal affect, but a selective COX-2 inhibitor attenuated the later temperature increase. This suggests the inducible COX-2 isozyme is predominantly responsible for the synthesis of PGE_2 and the late phase response of thermoregulatory neurons in the AH.

PGE_2 and the Prostaglandin E (EP) Receptors

The synthesis of PGE_2 is a multi-step, tightly regulated process that is the target of much investigation. Membrane bound Phospholipase A2 (PLA_2) cleaves phospholipids into arachadonic acid (AA). AA is converted by the COX isozymes and Peroxidase into the key intermediate PGH_2, which is transformed to PGE_2 by membrane-bound PGE synthase (mPGES-1). PGE_2 is subsequently released from the cell, where it acts though four subtypes of the EP receptor, identified as 1, 2, 3, and 4, based on their modes of signal transduction. The EP receptors have a seven-transmembrane domain that is linked to G-proteins which are involved in second messenger pathways (Siegel et al, 1999). Functionally, EP_1 increases intracellular Ca^{2+} levels, EP_2 and EP_4 increase cAMP, and EP_3 is inhibitory and lowers cAMP (Lazarus, 2006). The many targets of these pathways can lead to modification of ion channels, membrane receptors, and neurotransmitter release.

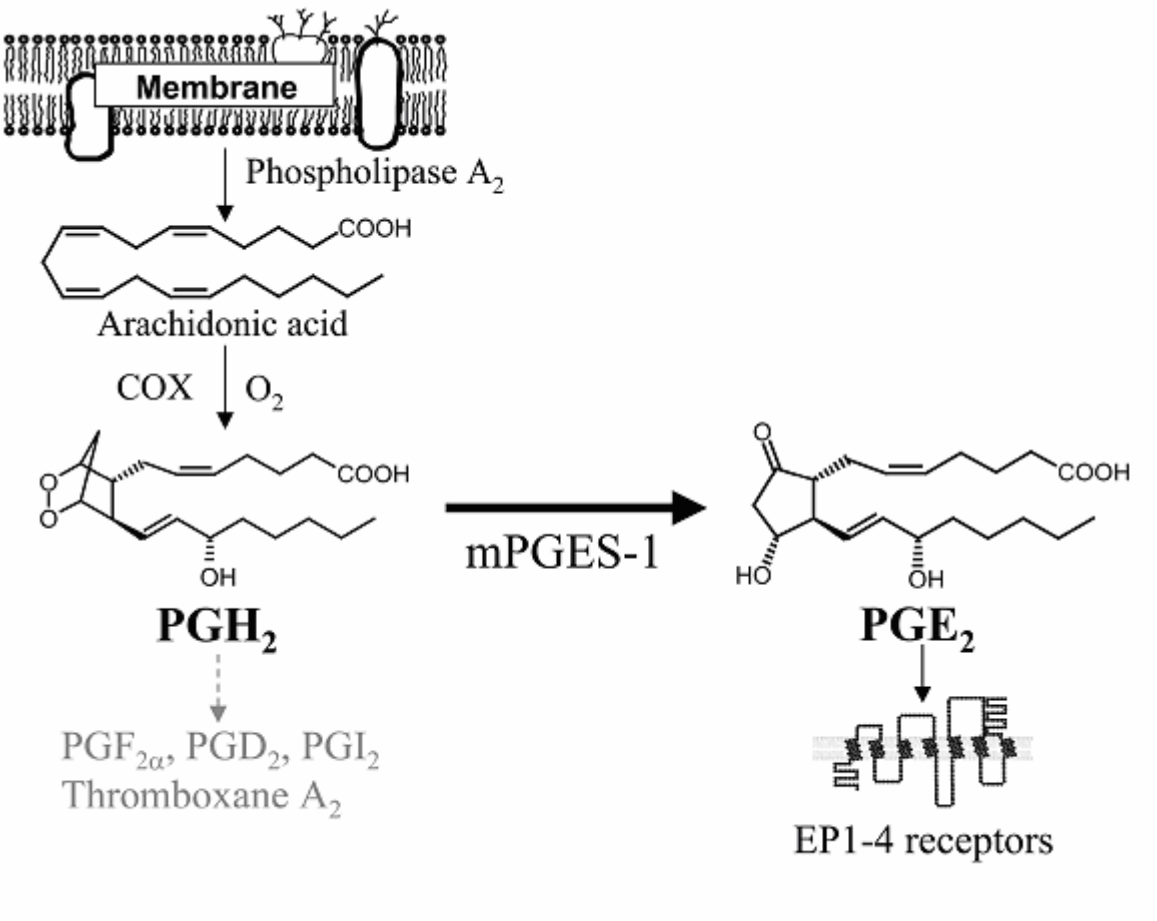

Figure 5, from Lazarus (2006). Cascade of PGE_2 production and key enzymes.

There remains some controversy about the signaling properties of PGE_2, mainly whether it predominantly functions as a local signal (paracrine) or is capable of widespread circulatory transport (endocrine). As a small, lipophillic molecule, it has been postulated to cross the blood brain barrier. In regards to the contemporary model of febrigenesis, most PGE_2 released by the Kc of the liver is presumed to directly stimulate the hepatic vagus. But some could enter the blood stream and enter the AH, through "leaky" areas of the blood-brain barrier, intensifying the fever initiated by the vagal afferents and its stimulation of NE release. Nonetheless, the observation that PGE_2 levels and the temperature rise in the AH induced by peripheral pyrogen exposure are inhibited

by intra-AH administration of COX inhibitors suggests that PGE_2 is predominantly generated locally in the hypothalamus, functioning as a paracrine signal (Blatteis et al., 2005).

Research on the EP receptors involved in the febrile response has been inconclusive. It was demonstrated that only mice lacking the EP_3 receptor via homologous recombination showed no febrile response when exposed to LPS (Ushikubi et al., 1998). Another knock-out study added more doubt when it was shown that both the EP_1 and EP_3 receptors were involved in an inflammatory febrile response (Oka et al., 2003). The discrepancies between these studies are likely due to differences in expression among various types of nervous tissue, but now more is known about the specific areas of the hypothalamus that these receptors are required for fever responses (Lazarus et al., 2007).

Through site-specific deletion of EP_3 receptors in certain nuclei of the rat hypothalamus, it has been revealed that expression of EP_3 receptors in the AH is crucial for the fever response (Lazarus et al., 2007). Agonists of the inhibitory EP_3 receptor have been shown to produce fever (Oka et al., 2003), presumably by an inhibition of warm-sensitive neurons due to decreased cAMP levels. In contrast, EP_4 receptors, are implicated in hypothermic responses (Lazarus et al, 2006). The differences in EP subtype expression and their contrasting thermal responses add another degree of complexity to febrigenesis. Due to their properties, it may be probable that EP_3 receptors are predominantly found on warm-sensitive neurons; likewise EP_4 receptors would be localized to insensitive neurons.

The regulation of COX-2 must be addressed to understand PGE_2 synthesis and its contribution to the distinct phases of fever. Of the COX enzymes, COX-1 is the constitutive form and COX-2 is the inducible type in most tissues, but constitutive levels are present in some neurons, dendrites, and astrocytes; its expression can be regulated quickly by inflammatory stimuli (Siegel et al., 1999). COX-2 appears to be the isozyme of central importance; in addition to almost completely abolishing fever, the administration of a COX-2 inhibitor after exposure to LPS will prevent the expression of Fos (a proto-oncogene that is up-regulated in response to activating stimuli) in anterior areas of the hypothalamus (Zhang et al., 2003). Less is known about the role of COX-1 and its involvement in fever production. It appears that COX-1 derived prostaglandins have unique roles in certain areas, especially the brainstem, perhaps as alternate routes to communicate with the CNS about inflammatory stimuli (Zhang et al., 2003). The expression of COX-1 and baseline PGE_2 production in these pathways along with COX-2 expression in the hypothalamus may be necessary to fully activate the autonomic nervous system during a febrile response.

Current Model of Fever in Response to an Immune Challenge

The current model suggests there is a dynamic interplay between NE and PGE_2 within the AH. The vagal-stimulated release of NE in the AH acts at two adrenorecpetors. The α1 AR is responsible for early phase febrigenesis, and directly augments the firing rates of warm-sensitive and insensitive neurons according to Hammel's model to bring about this temperature rise. NE acting at the α2 AR stimulates an initial hypothermia and via second messenger pathways induces COX-2 expression

and PGE_2 formation. The subsequent PGE_2 release acts at EP receptors, activating second messenger pathways bringing about late phase fever response via the inhibition of warm-sensitive neurons or activation of temperature insensitive neurons in accord with Hammel's model.

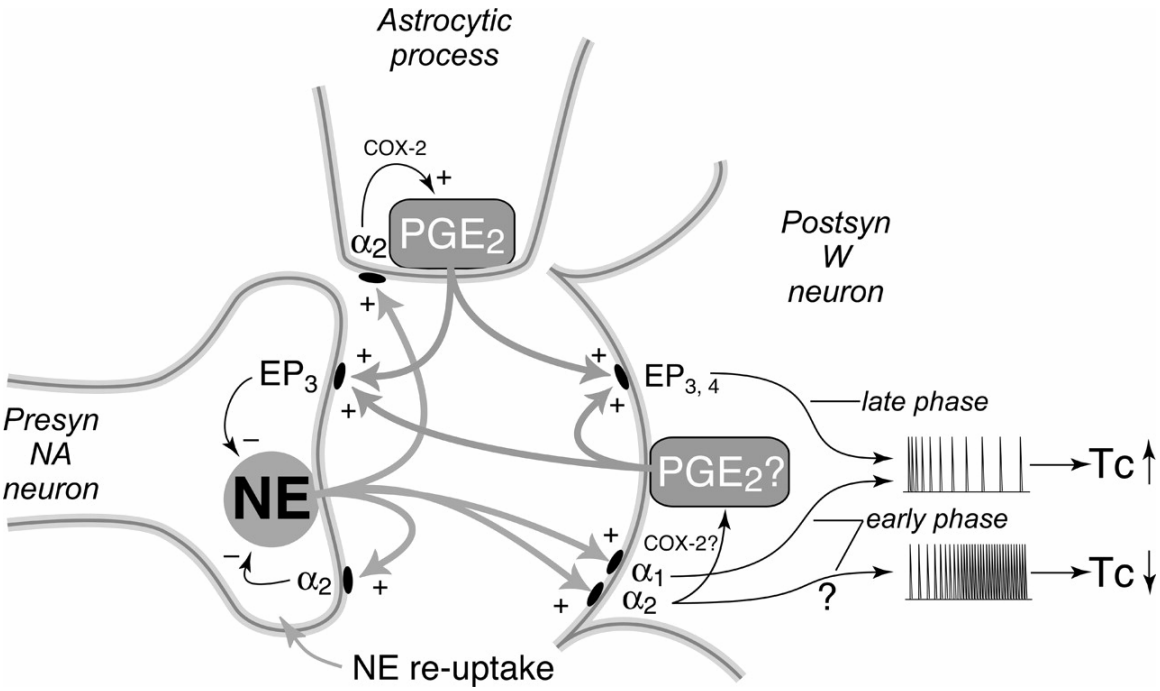

Figure 6, from Feleder et al. (2004). Schematic of NE and PGE_2 interaction in the Hypothalamus. See above text for details

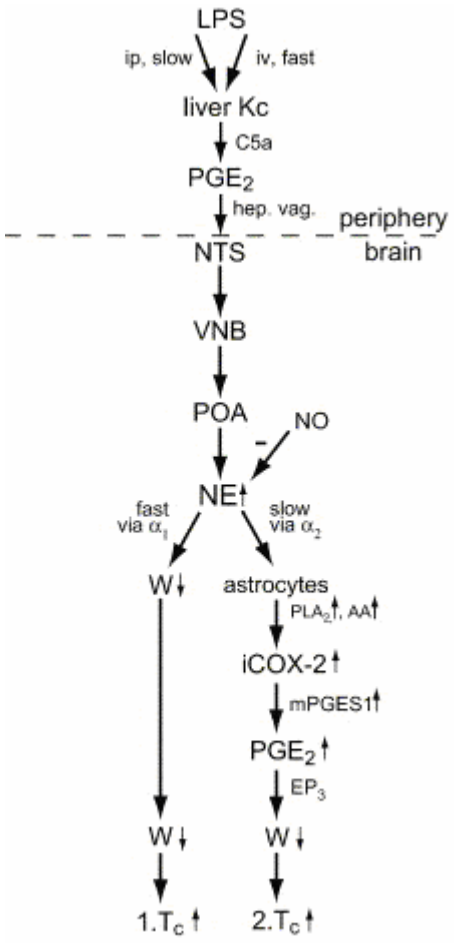

Figure 7, from Blatteis (2005). Diagram summarizing the proposed pathway of the biphasic febrile response mediated by NE and PGE_2. See text for details.

Project Summary and Objectives

This project was undertaken to provide more insight into the interaction of NE and PGE_2 within the AH during the fever. Specifically, I aim to confirm *in vivo* work demonstrating COX-2 independence of the α1 AR response and a COX-2 dependence of the late-phase α2 AR response using *in vitro* tissue slice preparations. Single-unit experiments are invaluable to the further neuronal understanding of thermoregulation during early and late-phase responses in the AH.

Hypotheses:

Treatment with the selective COX-2 inhibitor Meloxicam will have no affect on the responses of thermoregulatory neurons (a decrease and increase in warm-sensitive and insensitive neurons respectively) when treated with Cirazoline, the α1 AR agonist. Treatment with the same COX-2 inhibitor will abolish the late-phase thermal responses of these thermoregulatory neurons when treated with Clonidine, the α2 AR agonist, as it is likely a PGE_2 dependent process.

Methodology

To record the single-unit activity of AH neurons, brain tissue slices containing the AH were prepared from male Sprague-Dawley rats (Harlan; 100-150 grams), which were housed under standard conditions and provided unlimited food and water. Before each recording session, a rat was anesthetized using isoflurane and promptly decapitated, following procedures that have been approved by the Animal Care and Use Committee of the College of William and Mary. After dissection of the brain, a tissue block containing the hypothalamus was mounted on a vibratome and bathed in artificial cerebral spinal fluid (aCSF). One to three 400 or 500 μm thick tissue slices (saggital or coronal plane) were cut and then placed in an interface style recording chamber and allowed to equilibrate for an hour before recordings were made. Tissue slices were perfused with aCSF, which consisted of (in mM): 124 NaCl, 26 $NaHCO_3$, 10 glucose, 5 KCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$, and 1.24 KH_2PO_4 (Sigma Chemicals). The water-soluble, selective COX-2 inhibitor Meloxicam (Sigma Chemicals) was also dissolved in the aCSF solution at concentrations of 0.1 – 10 μM on the day of the recording. The perfusion medium was

gently aerated (95% O_2 – 5% CO_2) and allowed to gravity flow at 1-2 ml·minute^{-1} into the recording chamber (volume = 2 ml). Temperature was maintained ~36°C except for periodic warming and cooling to characterize the thermosensitivity of recorded neurons. A thermocouple was placed below the tissue slices to constantly monitor temperature.

To confirm the PGE_2 independence or dependence of the α1 and α2 AR response, Cirazoline or Clonidine (Sigma Chemicals) was diluted to 1 μM in the same aCSF + Meloxicam solution and oxygenated in a separate perfusion tube, which could be switched to the primary perfusion line in place of the aCSF + Meloxicam only medium by means of a valve control system. Thus at all times the tissue was exposed to aCSF + Meloxicam, and Clonidine or Cirazoline only introduced in the recording chamber during treatment periods.

To characterize the firing rate activity of individual neurons in the AH, single-unit recordings were made of electrical activity. Recordings were made using glass microelectrodes pulled to a tip diameter of ~1 μm and filled with a 3 M NaCl solution. Electrical activity was amplified and filtered using a Xcell-3 microelectrode amplifier and acquisition processor module system (FHC Inc.). An acceptable single-unit recording required potential amplitudes with a signal-to-noise ratio greater than 3:1. Firing rate was determined through the use of a Rate/Interval Monitor (FHC Inc.) and was continuously recorded, along with temperature, on a computer using Axoscope software (Molecular Devices). Once the activity of a single neuron had been isolated and firing rate recorded for an initial period of stability, temperature was varied 1-3 ^{o}C above and below the baseline temperature (~36 °C) by changing the input voltage to a thermoelectric heating assembly through which the perfusion media flowed before

entering the chamber. Neuronal thermosensitivity (impulses·s^{-1}·°C^{-1}) was later characterized by plotting firing rate as a function of temperature to determine the regression coefficient (*m*) of this plot. As in previous studies (Kelso et al., 1982; Fetsch et al., 2006), warm sensitivity was characterized by a regression coefficient of at least 0.8 impulses·s^{-1}·°C^{-1}. All other neurons in this study were classified as temperature insensitive.

Tissue temperature was again stabilized at a baseline level and the perfusion medium was switched from normal aCSF + Meloxicam to one containing Cirazoline or Clonidine. Perfusion with Cirazoline or Clonidine continued for 10 minutes or until a change in firing rate of at least 15% occurred. Cirazoline or Clonidine was then removed from the recording chamber by perfusion with normal aCSF + Meloxicam for at least 10 minutes. Occasionally, after a neuron's firing rate activity recovered to a stable level, a second perfusion with Cirazoline or Clonidine was performed.

To determine if there was a significant change in a neuron's firing rate in response to Cirazoline, firing rate measurements were collected for three segments (at 60 Hz): one minute before the beginning of perfusion with experimental solutions (Baseline), one minute during the peak of the response or immediately preceding the end of the experimental perfusion (Treatment), and one minute at the end of the following perfusion with previous solutions (Washout). For each segment, a mean and standard error were determined. A change in firing rate was considered significant between Baseline and Treatment if it was at least 15% and there was a significant difference at $p < 0.05$ (Student's t-test).

Neurons treated with Clonidine were analyzed similarly, but with another criterion in order to measure any late phase responses due to PGE_2. After the initial treatment of Clonidine, if possible, a period of at least thirty minutes was allowed to pass, since most evidence suggests the synthesis of PGE_2 requires approximately twenty to thirty minutes in response to an inflammatory signal (Simmons et al., 2004). Neurons that were able to be recorded for a prolonged period of time (such as one and a half to two hours) and recovered to a stable firing rate after initial Clonidine treatment were considered for late phase analysis. The washout firing rate served as a new baseline, and in a similar manner, cells were analyzed for any changes in firing rate during this late period. Responses from a previous study on the affects of PGE_2 in the AH (Ranels and Griffin, 2003) served as a guideline to examine any late phase responses.

After each recording, the location and depth of the electrode was noted on a section diagram adapted from an atlas of the rat brain (Paxinos and Watson, 1998). At the end of the recording session, tissue slices were removed from the chamber, fixed in a formalin solution, and placed in a 30% sucrose solution before being sectioned to a thickness of 40 - 50 μm. Sections were then mounted on gelatin coated slides and stained with giemsa to identify specific hypothalamic areas so the location of each recording within the AH could be reconfirmed.

Results

Cirazoline and COX-2 Inhibition

The firing rates of fifteen AH neurons were recorded during changes in temperature and perfusion with the α1 AR agonist Cirazoline (1μM) while exposed to the

COX-2 inhibitor Meloxicam (10 μM). With a criterion for warm sensitivity of $m \geq 0.8$ impulses$\cdot s^{-1} \cdot {}^{\circ}C^{-1}$, two neurons were classified as warm-sensitive while the majority (n = 13) were classified as temperature insensitive. **Figure 8** shows the percent change in firing rate responses to Cirazoline for each neuron, plotted as a function of the neuron's thermosensitivity. Nine of the temperature insensitive neurons showed significant increases in firing rate, greater that a 15% change during perfusion with Cirazoline. There were two insensitive neurons who did not respond significantly, with percent changes less than 15%. Two other insensitive neurons showed very dramatic increases, lying well outside the range of the rest of the data with over 650% changes. The two warm-sensitive neurons decreased their firing rate by about 40 and 100% (which reflects a complete shutdown of firing).

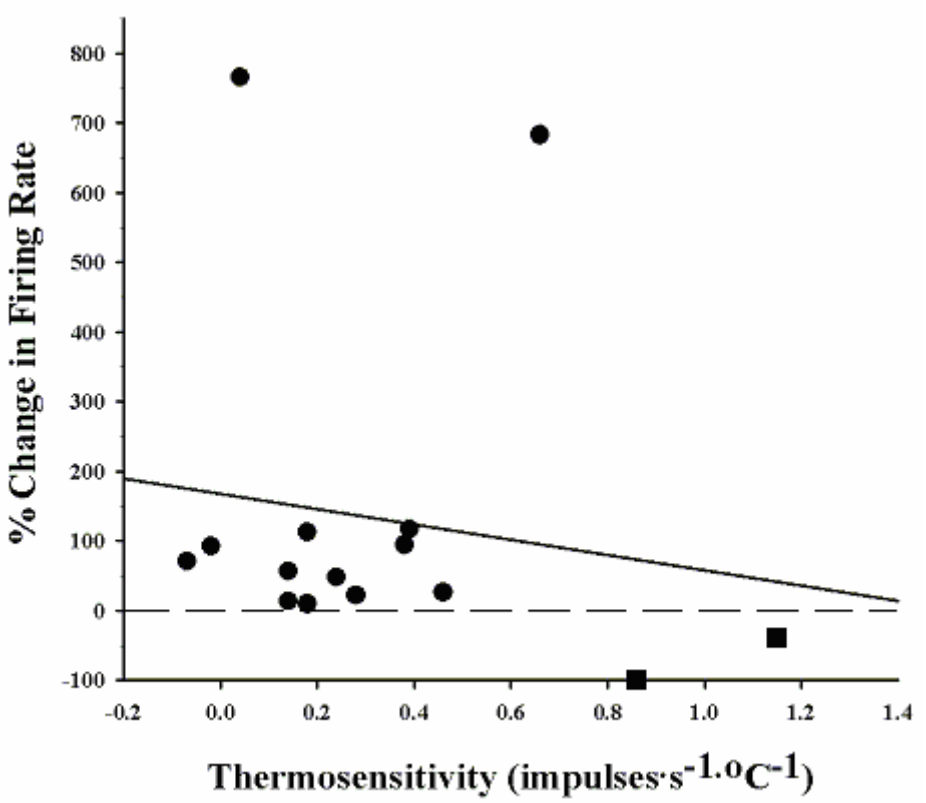

Figure 8. The firing rate responses of AH neurons to Cirazoline under COX-2 inhibition. The percent change in firing rate for all neurons ($N = 15$) in response to Cirazoline is plotted against thermosensitivity. A neuron was classified as warm-sensitive if it had a thermosensitivity ≥ 0.8 impulses$\cdot s^{-1.0}C^{-1}$, represented by squares ■ in the figure. All other neurons were classified as insensitive, represented as circles ●

Overall, the difference between baseline and treatment periods for the insensitive neurons was not significant at the $p < 0.05$ level, but was at $p < 0.10$ (Paired *t*–test $p = 0.08$). The two warm-sensitive neurons responded to Cirazoline with decreases in firing rate, but overall these differences were not significant (Paired *t*–test $p = 0.26$) compared to baseline. **Table 1** summarizes the changes in firing rates of these neurons during the three measurement conditions of baseline, treatment, and washout. As a population, the temperature insensitive neurons significantly increased their firing rates from a mean of 4.02 impulses$\cdot s^{-1}$ during baseline conditions, to 10.07 impulses$\cdot s^{-1}$ during perfusion with Cirazoline. In contrast, the mean firing rate of the warm-sensitive neurons decreased

from 2.84 impulses·s^{-1} to 1.46 impulses·s^{-1}. Seven temperature insensitive neurons did recover to a stable firing rate after Cirazoline treatment, the mean washout FR of these neurons was slightly lower than baseline at 2.08 impulses·s^{-1}. Among the warm-sensitive neurons, only one showed a stable recovery after Cirazoline treatment, it returned to a FR of 1.92 impulses·s^{-1}. The other warm-sensitive neuron remained inhibited for the duration of the recording after treatment. Because of the lack of warm-sensitive cells recorded, the standard error (SE) within this data group is very high.

Cirazoline *with* COX-2 inhibition (Meloxicam)

		Firing Rate-FR (impulses/sec ± SE)		
Cell Type	***N***	**Baseline**	**Treatment**	**Washout**
Insensitive	13	4.02 ± 0.66	10.07 ± 3.51*	2.08 ± 0.92
Warm	2	2.84 ± 2.06	1.46 ± 1.46	1.92 ± 1.92

(* denotes significant difference from Baseline Firing Rate. Paired *t*–test $p < 0.10$)

Table 1. Effects of Cirazoline with COX-2 inhibition on the firing rates of thermally classified AH neurons.

The firing rate activity of a temperature insensitive neuron with COX-2 inhibition during a change in temperature and perfusion with Cirazoline is shown in **Figure 9**. With respect to a variation in temperature (~ 4 °C), there was some correlation between firing rate, but it was not large enough to classify this neuron as warm-sensitive ($m = .39$). In response to perfusion with Cirazoline, firing rate increased from a baseline mean of 3.17 impulses·s^{-1} to 7.22 impulses·s^{-1}, peaking at a firing rate of 10.61 impulses·s^{-1} during

the middle of the treatment period. Within ten minutes from the time perfusion with Cirazoline was stopped, firing rate was much lower compared to baseline, 0.29 impulses·s^{-1}, and then died shortly thereafter.

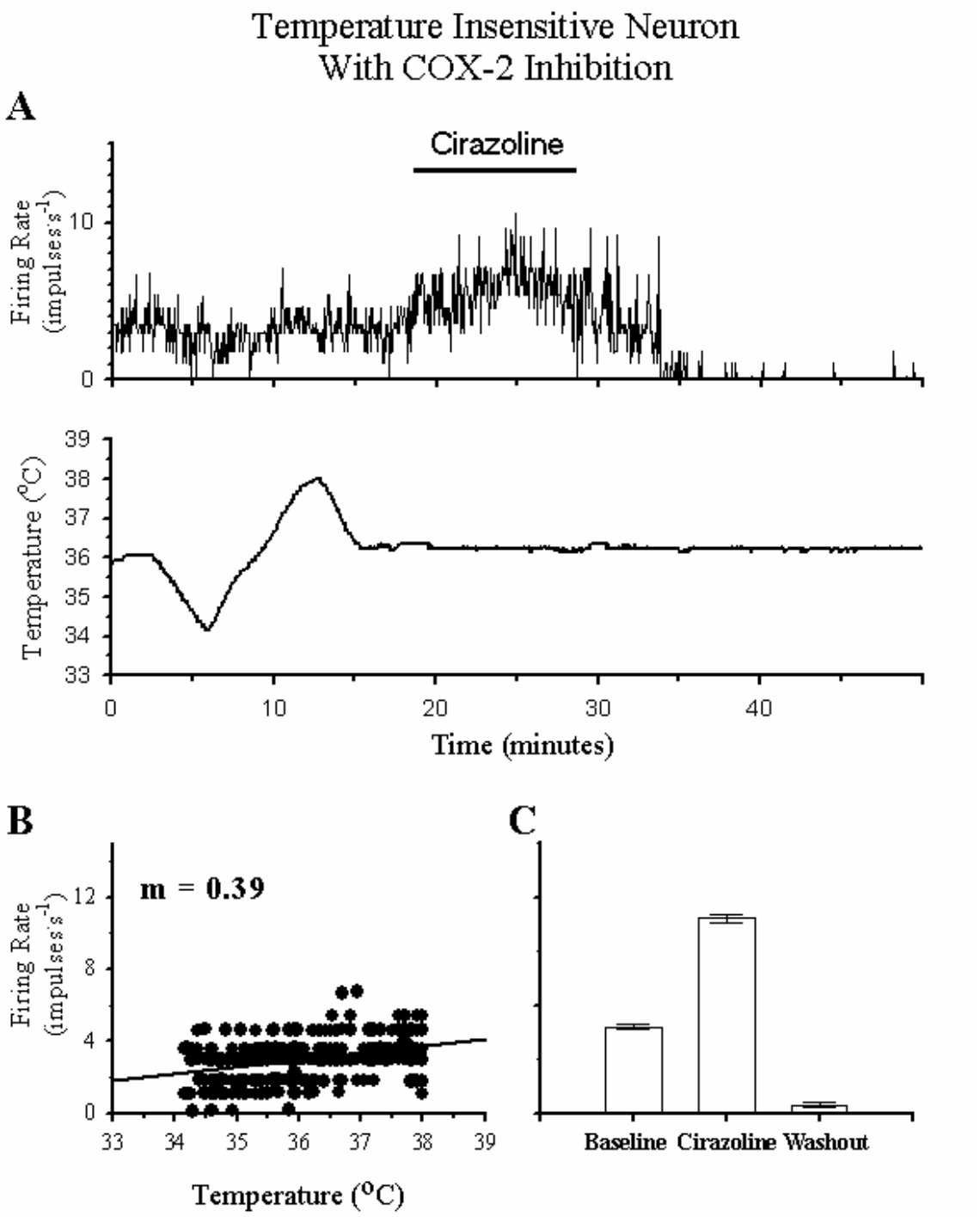

Figure 9. The effects of temperature and Cirazoline with COX-2 inhibition on the firing rate activity of an AH temperature insensitive neuron. **A** shows the firing rate of this neuron during changes in temperature and Cirazoline (1 μM; indicated by the solid bar above the graph). In **B**, firing rate (60 Hz) is plotted as a function of temperature. A linear regression is indicated by the solid line. In **C**, one minute segments of firing rate activity are plotted as individual bar graphs, just before perfusion with Cirazoline (Baseline; 3.17 ± 0.09), during the peak of the response (Cirazoline; 7.22 ± 0.15), and several minutes after Cirazoline perfusion had stopped (Washout; 0.28 ± 0.09).

The firing rate activity of a warm-sensitive neuron with COX-2 inhibition during a change in temperature and perfusion with Cirazoline is shown in **Figure 10**. With respect to a variation in temperature (~ 4 °C), there was a strong correlation between firing rate that was large enough to classify this neuron as warm-sensitive (m = 1.15). In response to perfusion with Cirazoline, firing rate decreased from a baseline mean of 4.50 impulses·s^{-1} to 2.52 impulses·s^{-1}. Within ten minutes from the time perfusion with Cirazoline was stopped, firing rate had returned to a stable level, with a mean firing rate of 3.43 impulses·s^{-1}. This washout firing rate remained stable for about twenty minutes before the recording was ended.

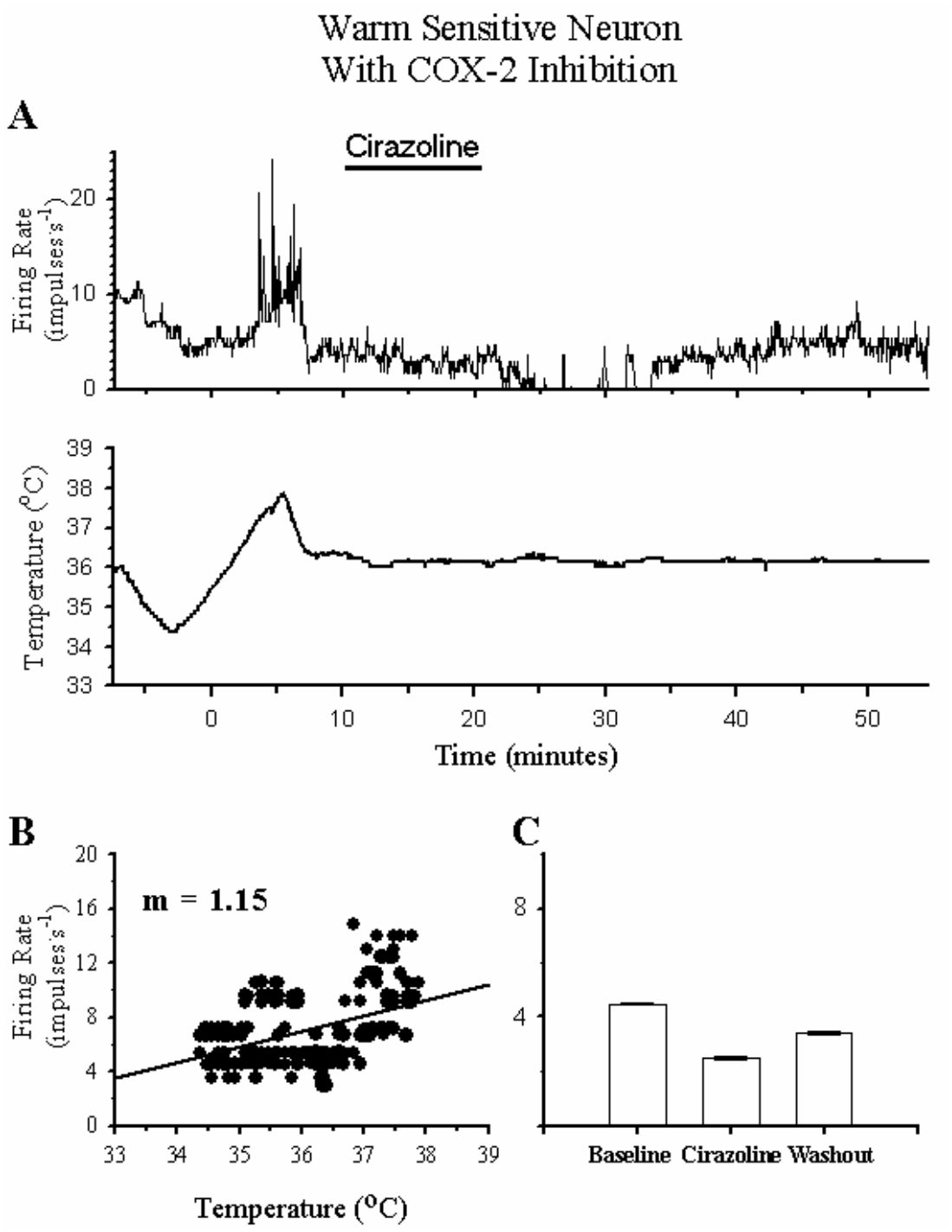

Figure 10. The effects of temperature and Cirazoline with COX-2 inhibition on the firing rate activity of a an AH temperature insensitive neuron. **A** shows the firing rate of this neuron during changes in temperature and Cirazoline (1 μM; indicated by the solid bar above the graph). In **B**, firing rate (60 Hz) is plotted as a function of temperature. A linear regression is indicated by the solid line. In **C**, one minute segments of firing rate activity are plotted as individual bar graphs, just before perfusion with Cirazoline (Baseline; 4.50 ± 0.06), during the peak of the response (Cirazoline; 2.52 ± 0.07), and several minutes after Cirazoline perfusion had stopped (Washout; 3.43 ± 0.05).

It is important to compare this data with results from a similar study analyzing the effects of Cirazoline without COX-2 inhibition on AH neurons (Imbery et al., 2008). Again, the criterion for warm sensitivity was $m \geq 0.8$ impulses$\cdot s^{-1} \cdot °C^{-1}$, all other neurons were classified as temperature insensitive. **Table 2** summarizes these changes in FR during each of the three measurement periods. The population of temperature insensitive neurons significantly increased their firing rates from a mean of 3.4 impulses$\cdot s^{-1}$ during baseline conditions, to 10.5 impulses$\cdot s^{-1}$ during perfusion with Cirazoline. In contrast, the mean firing rate of the warm-sensitive neurons significantly decreased from 3.7 impulses$\cdot s^{-1}$ to 0.4 impulses$\cdot s^{-1}$. Comparisons between the responses of Cirazoline with COX-2 inhibition and Cirazoline only will be addressed in the discussion for further analysis.

Cirazoline *without* COX-2 inhibition

		Firing Rate-FR (impulses/sec ± SE)		
Cell Type	***N***	**Baseline**	**Treatment**	**Washout**
Insensitive	37	3.41 ± 0.38	10.49 ± 1.40*	2.68 ± 0.84
Warm	12	3.71 ± 0.50	0.44 ± 0.19*	0.41 ± 0.00

(* denotes significant difference from Baseline Firing Rate. Paired *t*–test $p < 0.05$)

Table 2. Effects of Cirazoline only on the firing rates of thermally classified AH neurons.

Figure 11 provides a detailed anatomical view of the location of each recording made with Cirazoline and COX-2 inhibition in this study. The majority of recordings were from neurons in the medial preoptic and anterior hypothalamic areas, with a few

recordings from neurons in more lateral and dorsal areas of the areas of the hypothalamus. Six temperature insensitive neurons were located in close proximity to the midline, and the remaining seven where in more lateral areas left and right. The two warm-sensitive neurons were also located in lateral areas of the anterior hypothalamus.

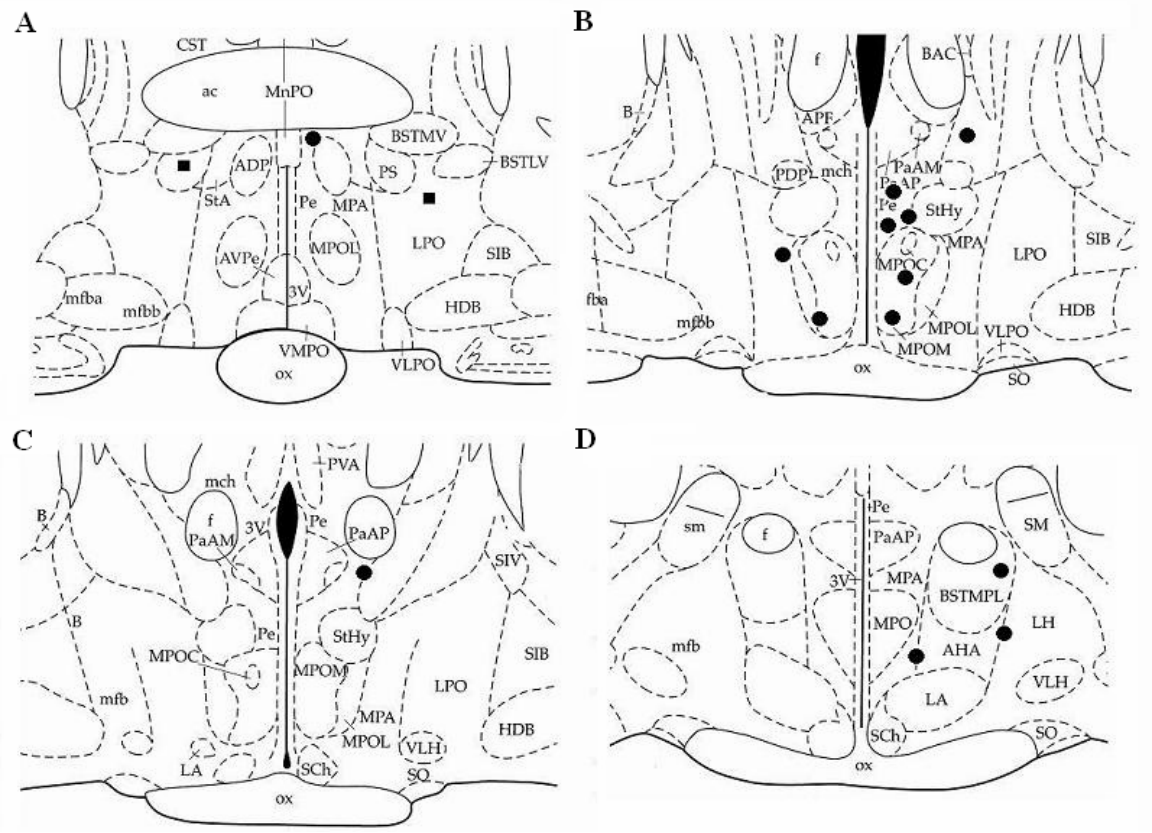

Figure 11. The electrode locations for recordings of single neuron activity in response to temperature and Cirazoline with COX-2 inhibition. Section diagrams are shown in the coronal plane and ordered from rostral to caudal, beginning with the upper left section and moving across each row. Distance from bregma: A = – 0.5 mm; B = – 0.7 mm; C = – 1.0 mm; D = – 1.3 mm. Sections were adapted from an atlas of the rat brain (Paxinos and Watson, 1998). Circles ● = insensitive neurons, Squares ■ = warm-sensitive neurons. Key abbreviations for reference: 3V, third ventricle; ac, anterior commissure; AHA, anterior hypothalamic area; f, fornix; LH, lateral hypothalamus; LPO, lateral preoptic area; MnPO, median preoptic nucleus; MPO, medial preoptic nucleus; MPA, medial preoptic area; mfb, median forebrain bundle; ox, optic chiasm; Pe, periventricular nucleus; Sch, suprachiasmatic nucleus; StHy, striohypothalamic nuc.; VLPO, ventrolateral preoptic area; VMPO, ventromedial preoptic area.

Clonidine and COX-2 Inhibition

The firing rates of thirty AH neurons were recorded during changes in temperature and perfusion with the α2 AR agonist Clonidine (1μM) while exposed to the COX-2 inhibitor Meloxicam (0.1-10 μM). With a criterion for warm sensitivity of $m \geq 0.8$ impulses$\cdot s^{-1} \cdot °C^{-1}$, three neurons were classified as warm-sensitive while the majority (n = 27) were classified as temperature insensitive. **Figure 12** shows the percent change in firing rate responses to Clonidine for each neuron, plotted as a function of the neuron's thermosensitivity. Three of the temperature insensitive neurons showed an increase in firing rate during perfusion with Clonidine, in contrast to the majority who showed a significant decrease in firing rate. Two warm-sensitive neurons responded to Clonidine with a significant increase in firing rate, approximately a 100% change. One warm-sensitive cell, whose had thermosensitivity $m = 3.96$, was omitted from the graph as an outlier because it showed an almost complete shutdown in firing rate, thus completely inverting the regression line.

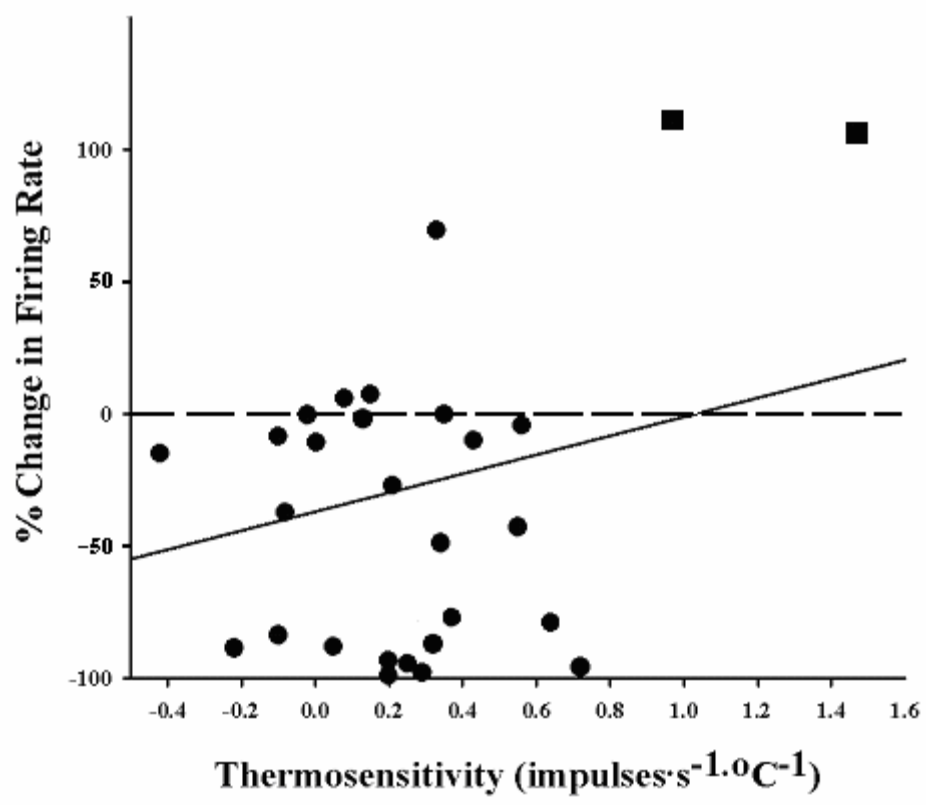

Figure 12. The firing rate responses of AH neurons to Clonidine under COX-2 inhibition. The percent change in firing rate for all neurons ($N = 30$) in response to Clonidine is plotted against thermosensitivity. A neuron was classified as warm-sensitive if it had a thermosensitivity $m \geq 0.8$ impulses·$s^{-1.0}C^{-1}$, represented by squares ■ in the figure. All other neurons were classified as insensitive, represented as circles ●

Table 3 summarizes the changes in firing rates of these neurons during the three measurement conditions of baseline, treatment, and washout. As a population, the temperature insensitive neurons significantly decreased their firing rates from a mean of 2.80 impulses·s^{-1} during baseline to 1.90 impulses·s^{-1} during perfusion with Clonidine. In contrast, the mean firing rate of the warm-sensitive neurons increased from 4.51 impulses·s^{-1} to 6.83 impulses·s^{-1}, but this increase was not statistically significant (Paired *t*–test $p = 0.38$). Twenty temperature insensitive neurons did recover to a stable firing rate after Clonidine treatment, the mean washout FR of these neurons was slightly lower than baseline at 1.92 impulses·s^{-1}. Among the warm-sensitive neurons, only one

showed a stable recovery after Clonidine treatment, it returned to a FR of 1.15 impulses·s^{-1}. The other warm-sensitive neurons did not return to a stable FR after treatment. Because of the lack of warm-sensitive cells recorded, the SE within this data group again is very high and there is not enough statistical power to discern any significant differences.

Clonidine with COX-2 inhibition (Meloxicam)

		Firing Rate (impulses/sec ± SE)		
Cell Type	***N***	**Baseline**	**Treatment**	**Washout**
Insensitive	27	2.80 +/- 0.35	1.90 +/- 0.46*	1.92 +/- 0.54
Warm	3	4.51 +/- 1.90	6.83 +/- 5.03	0.38 +/- 0.38

(* denotes significant difference from Baseline Firing Rate. Paired *t*–test $p < 0.05$)

Table 3. Effects of Clonidine with COX-2 inhibition on the firing rates of thermally classified AH neurons.

There were nine neurons, all of which were temperature insensitive, recorded for a period of at least thirty minutes after the initial Clonidine treatment that were analyzed for a late phase PGE_2 response. None of these neurons showed any significant increase in firing rate compared to the washout firing rate. Refer to **Figure 13,** highlighting this period of stability after Clonidine treatment.

The firing rate activity of a temperature insensitive neuron with COX-2 inhibition during a change in temperature and perfusion with Clonidine (1 μM) is shown in **Figure 13**. With respect to a variation in temperature (~ 4 °C), there was little correlation with firing rate (m = .03), so it was classified as temperature insensitive. In response to perfusion with Clonidine, firing rate decreased from a baseline mean of 2.22 impulses·s^{-1}

to 1.97 impulses·s^{-1}. This decrease in firing rate was not statistically significant (paired *t*–test $p = 0.13$). Once Clonidine perfusion was stopped, the firing rate returned to a mean of 1.40 impulses·s^{-1} and was very stable. The recording continued on for approximately 100 minutes after the end of treatment, and no significant late phase increase in firing rate was observed.

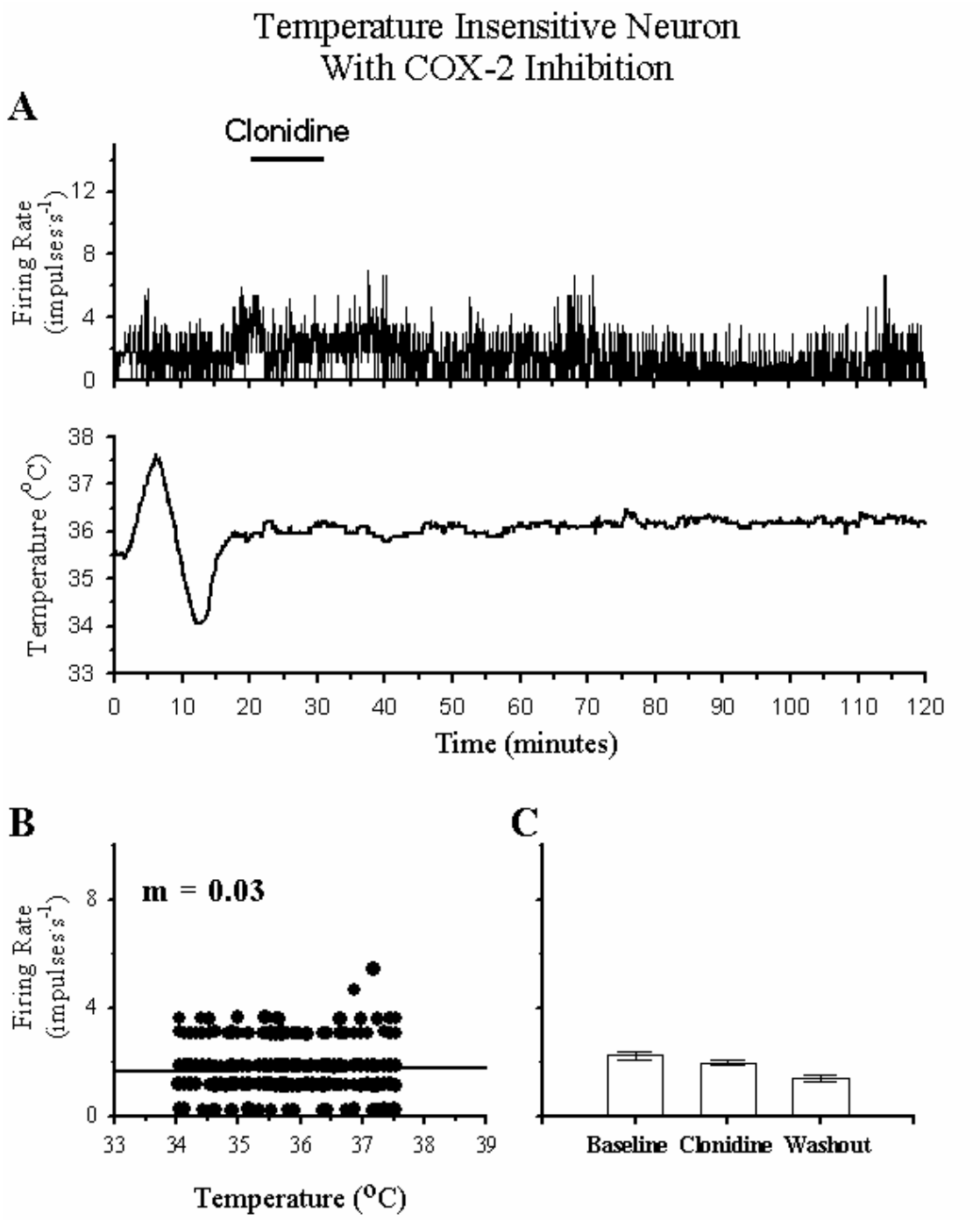

Figure 13. The effects of temperature and Clonidine with COX-2 inhibition on the firing rate activity of an AH temperature insensitive neuron. **A** shows the firing rate of this neuron during changes in temperature and Clonidine (1 μM; indicated by the solid bar above the graph). In **B**, firing rate (60 Hz) is plotted as a function of temperature. A linear regression is indicated by the solid line. In **C**, one minute segments of firing rate activity are plotted as individual bar graphs, just before perfusion with Clonidine (Baseline; 2.22 ± 0.14), during the peak of the response (Clonidine; 1.97 ± 0.7), and several minutes after Clonidine perfusion had stopped (Washout; 1.40 ± 0.1).

For comparison with **Figure 13**, the firing rate activity of an insensitive neuron treated with Clonidine (1 μM) but no COX-2 inhibition is shown in **Figure 14**. With respect to a variation in temperature (~ 4 °C), there was a slight correlation with firing rate (m = -0.36), showing some cold sensitivity, but it is still classified as a temperature insensitive neuron. A five minute perfusion with Clonidine produced a significant decrease in firing rate from a baseline of 4.98 impulses·s^{-1} to 1.50 impulses·s^{-} 1 (paired t–test p = 0.00). After ten minutes, the cell returned to a mean washout firing rate of 6.57 impulses·s^{-1}. At approximately thirty minutes into the recording, twenty minutes since the Clonidine treatment, the firing rate began to drastically increase, reaching a peak firing rate of 45 impulses·s^{-1}. This plateau response lasted about eight minutes before the recording was stopped, typical of an insensitive neuron responding to PGE_2.

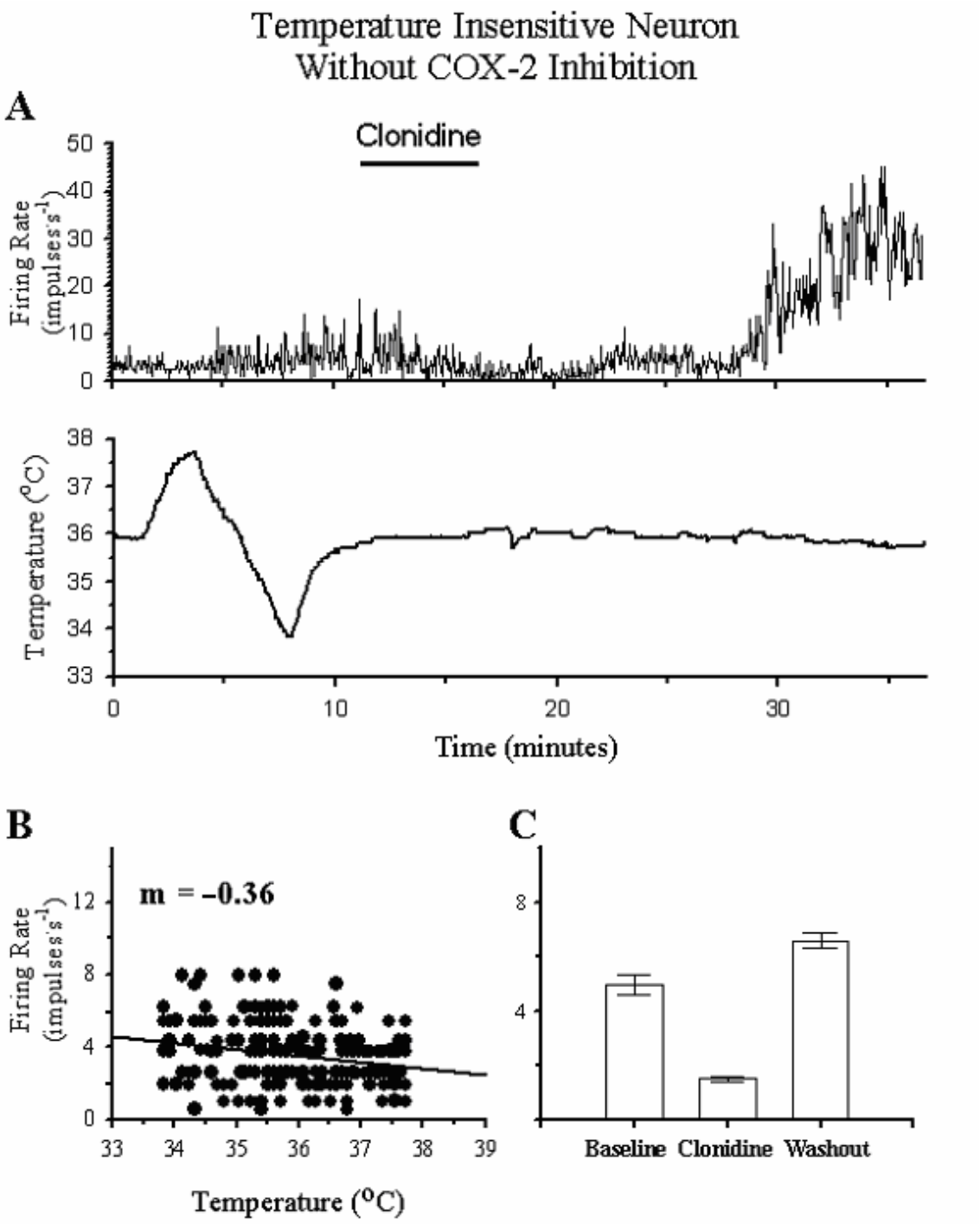

Figure 14. The effects of temperature and Clonidine without COX-2 inhibition on the firing rate activity of an AH insensitive neuron. **A** shows the firing rate of this neuron during changes in temperature and Clonidine (1μM; indicated by the solid bar above the graph). In **B**, firing rate is plotted as a function of temperature. A linear regression is indicated by the solid line. In **C**, one minute segments of firing rate activity are plotted as individual bar graphs, just before perfusion with Clonidine (Baseline; 4.98 ± 0.37), during the peak of the response (Clonidine; 1.50 ± 0.09), and several minutes after Clonidine perfusion had stopped (Washout; 6.58 ± 0.26). Note after thirty minutes, there is a sustained increase in firing rate, reaching a peak of 45 impulses·s^{-1}

Figure 15 displays a detailed anatomical view of the location of each recording presented in this study. While a majority of recordings were made from neurons in the medial preoptic and anterior hypothalamic areas, a few recordings extended well into posterior regions of the hypothalamus. There was no specific area of the hypothalamus in which temperature insensitive or warm-sensitive neurons were found in higher proportions, though warm-sensitive neurons were located in relative proximity to the fornix.

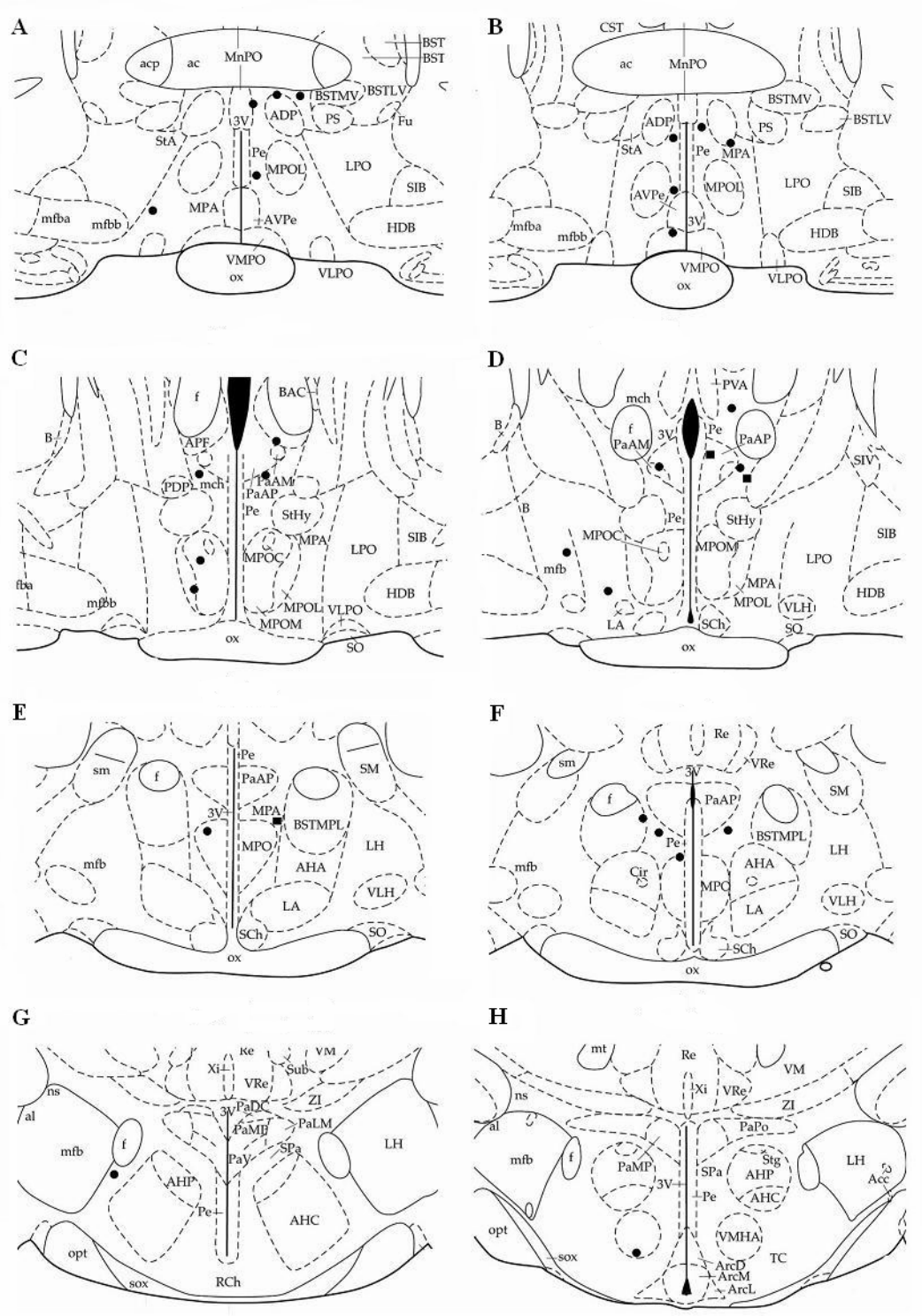

Figure 15. Electrode locations for recordings of single neuron activity in response to temperature and Cirazoline with COX-2 inhibition. Section diagrams are shown in the coronal plane and ordered from rostral to caudal, beginning with the upper left section and moving across each row. Distance from bregma: A = – 0.5 mm; B = – 0.7 mm; C = – 1.0 mm; D = – 1.3 mm. Sections were adapted from an atlas of the rat brain (Paxinos and Watson, 1998). Circles ● = insensitive neurons, Squares ■ = warm-sensitive neurons. Key abbreviations same as in **Figure 11**.

Discussion

The fever response is an important mechanism in the body's line of defense against immune system challenges. Traditionally, it was believed that PGE_2 was the critical fever signal, produced either via circulating or fixed mononuclear phagocytes in the periphery. Once transported in the bloodstream, PGE_2 would enter the AH by means of transport through permeable areas of the blood brain barrier (Blatteis, 1997). However, this mechanism is rather slow, and it could not account for the fact that COX-2 expression actually appeared much later than the actual onset of fever. This suggests that a mechanism independent of PGE_2 production was driving the initial fever response.

Recent *in vivo* research instead demonstrates that it is hepatic vagal afferents which promptly convey information from the immune system to the hypothalamus to initiate a fever in response to a peripheral LPS challenge. Via this neural route, it is the subsequent release of NE within the AH which is a critical fever mediator through its action at α1 and α2 ARs of thermoregulatory neurons (Feleder et al., 2007). Stimulation of α1 ARs gives rise to a prompt temperature rise independent of PGE_2, whereas activation of α2 ARs yields a biphasic response, producing an initial hypothermia and later generating a temperature rise that is dependent on COX-2 derived PGE_2 synthesis (Feleder et al., 2004). This twofold mechanism can better account for the prompt, initial temperature rise and the slower elevation of PGE_2 levels. Therefore, NE acting at α1 ARs must directly augment the firing rates of thermoregulatory neurons to quickly generate heat production, while the simultaneous activation of α2 ARs would initiate second messenger pathways responsible for downstream PGE_2 synthesis. In this sense,

NE is more a fever initiator, whereas PGE_2 acts later through EP receptors and is responsible for sustaining this hyperthermia.

This study analyzed the responses of thermoregulatory neurons within an AH tissue slice preparation in the presence of selective α1 and α2 AR agonists while production of COX-2 derived PGE_2 was inhibited. It is important to complement *in vivo* research with *in vitro* methodology to better understand the properties of thermoregulatory neurons driving these NE-mediated thermal responses. Confirmation of the PGE_2-independence and PGE_2-dependence of these two respective pathways can provide more support for the neural mechanism of fever induction and a better understanding of thermoregulatory synaptic networks in the AH.

Hammel's (1965) model outlines an elegant six neuron synaptic network in the AH to account for changes of a thermoregulatory set-point. It is centered on mutually antagonistic synapses of warm-sensitive and temperature insensitive neurons with effector neurons that control thermoregulatory responses. Warm-sensitive neurons synaptically excite heat-loss effector neurons and inhibit heat-production effector neurons whereas the temperature insensitive neurons inhibit heat-loss effector neurons and excite heat-production effector neurons. Thus, the inhibition of warm-sensitive neurons and excitation of temperature insensitive neurons would produce a hyperthermic shift of set-point temperature. In contrast, excitation of warm-sensitive neurons and inhibition of temperature insensitive neurons would drive the set-point into a hypothermic range.

In this study, perfusion of the α1 AR agonist with COX-2 inhibition resulted in firing rate changes in agreement with Hammel's model. The majority of temperature insensitive neurons showed a rapid increase in firing rate when exposed to Cirazoline,

whereas both warm-sensitive neurons exhibited a long term shutdown. For comparison, the data from a previous study analyzing Cirazoline's effects without COX-2 inhibition (Imbery et al., 2008) is included. Although the sample size for this study is noticeably smaller and lacking in warm-sensitive cells, the results are very similar. Between both groups, the most striking similarity is the response of the temperature insensitive neurons, each reaching average treatment firing rates near 10 impulses$\cdot s^{-1}$, the slight difference not being significant. Though there is a significant difference between the degree of inhibition for warm-sensitive neurons from these two studies, it is likely due to the low sample size in the present study. The lack of warm-sensitive cells in this study may be a result of more recordings being made near the third ventricle (midline) as opposed to more lateral areas near the fornix, where there are greater proportions of warm-sensitive neurons (Griffin et al., 2001).

Together, these findings suggest that the effect of NE at the α1 AR is responsible for directly modifying the firing rate of temperature sensitive and warm-sensitive neurons to drive set-point into a hyperthermic range. It also supports evidence that the α1 AR is widely localized on neurons within the AH, but the specific subtype remains to be identified. Since it is a rapid response, it presumably involves modification of ion channel conductances, via a mechanism related to the α1 AR stimulation of PLC production, perhaps involving calcium. Further intracellular analysis will be needed to discern the specific conductances involved. Generally in the brain, α1 activation is excitatory and results in slow depolarization linked to the inhibition of potassium channels (Purves et al., 2004). As well, molecular evidence suggests that the α1 mediates an increase in sodium pump activity (Mallick et al., 2000). Given that the α1 AR is

responsible for the initial temperature rise and not maintaining later phases, the hyperthermic affect of Cirazoline likely attenuates due to receptor desensitization after continuous stimulation. But by the time the α1 AR response attenuates, the levels of PGE_2 will have risen due to α2 AR activation, thus bringing about the second phase of fever maintenance.

Perfusion of the α2 AR agonist Clonidine produces a biphasic response, a hypothermia followed by late phase hyperthermia. Hammel's model predicts that this initial hypothermia would be driven by an increase in firing rate of warm-sensitive neurons and a decrease in firing rate of temperature insensitive neurons, just the opposite of what was observed with Cirazoline α1 AR stimulation. The findings from this study support Clondine's direct hypothermic effects. Despite COX-2 inhibition, twenty-four out of twenty-seven insensitive neurons decreased their firing rate in response to Clonidine. Two warm-sensitive neurons significantly increased their firing rate in response to Clondine, but there was one warm-sensitive neuron which did show a drastic decrease in firing rate. It is difficult to reach an accurate conclusion of Clonidine's affect on warm-sensitive neurons with a small sample size of three. As the case with the Cirazoline COX-2 inhibition data, the lack of warm-sensitive cells recorded in this study may be attributed to recordings being made closer to the third ventricle. It does not appear that the COX-2 inhibitor Meloxicam was decreasing the probability of recording from warm-sensitive neurons or that it reduced the inherent thermosensitivities of these neurons, though more controls in this regard could be performed for confirmation.

This data suggests that Clonidine's action at the α2 AR results in direct modification of ion conductances in thermoregulatory neurons to establish a hypothermic

set-point. How this occurs remains to be elucidated, but intracellular recordings could provide answers in this regard. In contrast to the α1 AR, α2 stimulation usually results in a slow hyperpolarization due to activation of potassium channels (Purves et al., 2004), and these antagonistic affects may account for the different thermal responses they mediate. But the question remains about these two opposite thermal responses which would initially be competing when NE is released in the AH. Presumably, the hypothermic α2 affect is masked by simultaneous, more profound α1 stimulation, producing a net hyperthermia. This could be tested by administration of Clonidine and Cirazoline together or either NE directly. The NE hyperthermic effects should manifest and occur in succession; the first (Cirazoline) rapid in onset, α1 AR mediated and the second (Clonidine) delayed, α2 AR mediated and PGE_2-dependent.

In this study, nine temperature insensitive neurons were recorded for a period of at least thirty minutes after Clonidine administration to examine late phase response. If PGE_2 is responsible for driving a late phase hyperthermia, Hammel's model predicts an increase and decrease in firing rate of temperature insensitive and warm-sensitive neurons, respectively, to shift set-point, similar to what is observed for Cirazoline. In all of these neurons, none showed a significant, prolonged rise in firing rate that is typical of a PGE_2 response (Ranels and Griffin, 2003). Although this study is limited since PGE_2 levels were not actually measured, these findings provide more evidence that a COX-2 dependent PGE_2 pathway is indeed responsible for producing the late phase α2 response. The interaction between NE and PGE_2 during fever is well documented (Sehic et al., 1996), and the likely intermediary is cAMP. Activation of the α2 AR is often coupled with inhibition of adenylate cyclase, thus decreased cAMP production may be leading to

increased PGE_2 synthesis and release, though an exact mechanism is unknown. PGE_2 would then exert direct effects on thermoregulatory neurons via an EP_3 or EP_4 mediated pathway to raise the set-point.

Together, this study has contributed more support for a biphasic fever mechanism mediated by NE within the AH thermoregulatory network described by Hammel (1965). The first phase is mediated rapidly by the α1 AR, directly augmenting the firing rate of thermoregulatory neurons in accordance with Hammel's model in a PGE_2-independent pathway. As well, the initial α2 AR stimulation produces a PGE_2 -independent hypothermia via contrasting responses of these thermoregulatory neurons, but this is likely masked by the simultaneous α1 AR pathway. The late phase is dependent on α2 AR activation of downstream COX-2 derived PGE_2 production, which then acts to maintain the set-point temperature within a hyperthermic range.

Acknowledgments

I would like to thank the Dr. Griffin for his continual support of my project and helping to stimulate my interests in research. It has been a privilege to work in your lab and I will always be thankful for the opportunity you have given me. I am grateful for the services of Drs. Coleman and Landino on my committee, two great professors whom I have especially enjoyed taking classes with. To members of the lab, many thanks for all the help and fun along the way!

This work was supported in part by a Howard Hughes Medical Institute grant through the Undergraduate Science Program to the College of William and primarily by a grant to Dr. Griffin from the National Science Foundation: IBN-9983624, the National Institutes of Health: NS053794 Mary.

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